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A New Sulfur-Reducing, Extremely Thermophilic Eubacterium from a Submarine Thermal Vent†

SHIMSHON BELKIN,[1] CARL O. WIRSEN,[2] AND HOLGER W. JANNASCH[2]*

Membrane Bioenergetics Group, Department of Physiology/Anatomy, University of California, Berkeley, California 94720,[1] and Department of Biology, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543[2]

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A newly described bacterial isolate, designated strain NS-E, differs from presently known extremely thermophilic bacteria in various characteristics. It is a strictly heterotrophic eubacterium of marine origin and has a temperature range for growth of 50 to 95°C with an optimum at 77°C and a pH of 7.5. Its DNA base composition is 41.3 mol% guanine + cytosine. It is obligately anaerobic, utilizes various sugars as well as yeast extract, and reduces elemental sulfur facultatively to hydrogen sulfide. In 24-h cultures cell densities are up to fourfold higher in the presence than in the absence of elemental sulfur. Sulfide concentrations of 1.0 and 10.0 mM limit growth by 65 and 95%, respectively. Oxygen sensitivity is apparent only at or above that range of temperature at which growth occurs.

The biology of thermophilic bacteria has elicited considerable interest over the last decade. Particularly exciting were the reports concerning extremely thermophilic species, their ecological role (26), their potential biotechnological applications (21), and numerous problems associated with life at temperatures as high as 100°C and above (1, 5, 15, 30).

Earlier work on extremely thermophilic bacteria concentrated mainly on aerobic species (5). More recently, several reports have been published which describe obligately anaerobic extreme thermophiles. These can be divided into two groups: (i) the extremely thermophilic archaebacteria, mainly those known as the thermoacidophiles (2, 7, 24, 35–38) and a few methanogens (11, 25), and (ii) extremely thermophilic eubacteria, some belonging to various new or known genera (4, 20, 29, 33, 34) and others which have not been classified (28).

In the second group, recent extremely thermophilic isolates include various nonsporeforming, mostly sugar-fermenting bacteria, all of them with optimal growth temperatures of 65 to 70°C. *Thermoanaerobium brockii* (34), *Thermoanaerobacter ethanolicus* (29), *Thermobacteroides acetoethylicus* (4), and *Thermodesulfobacterium commune* (33) are four new species which have been well characterized.

We present here the preliminary description of a marine, obligately anaerobic, sugar-fermenting eubacterial isolate which cannot be classified under any of the above genera.

MATERIALS AND METHODS

Isolation. Anoxic sediment and water samples were collected at a shallow (6-m) submarine hot spring near Lucrino, Bay of Naples, Italy, and were stored at room temperature under N_2.

Nitrogen-flushed, serum-capped 17-ml test tubes (Hungate type; Bellco Glass, Inc., Vineland, N.J.) with 10 ml of 2216 marine broth (Difco Laboratories, Detroit, Mich.) were inoculated and incubated at 75°C. After 2 to 4 days, bacterial growth occurred in several tubes. Purifications were successfully carried out by streaking on agar (3%) plates of the same medium, which were incubated at 75°C under N_2 in stainless-steel anaerobic jars (model AJ-2; Torsion Balance Co., Clifton, N.J.) in the presence of anhydrous $CaSO_4$. Colonies appeared within 2 to 4 days. One strain isolated in this manner, designated NS-E, is described in this paper.

Culture conditions. Strain NS-E was routinely grown in marine broth diluted to half strength in synthetic seawater (Turks Islands Salts, see reference 16). For some of the studies, an artificial seawater medium was used as described by Ruby et al. (19), with or without added organic compounds. In both cases, a vitamin mix (thiamine hydrochloride, biotin, and B_{12}, 40 μg of each liter^{-1}), resazurin (0.8 mg liter^{-1}), and PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer (20 mM, pH 7.5) were added.

Liquid medium (10 ml) with or without 100 mg of steam-autoclaved (110°C) elemental sulfur (sublimed "sulfur flower" [USP]; Fisher Scientific Co., Pittsburgh, Pa.) was placed in Hungate tubes and flushed for 10 min with N_2 gas passed though a heated copper furnace. Sodium thioglycolate was then injected to a final concentration of 0.2 g liter^{-1}, and the tubes were preincubated at 77°C. After approximately 1 h, during which time the resazurin turned colorless, the medium was inoculated to 10^6 cells ml^{-1} from an overnight culture, and the tubes were incubated at 77°C without shaking.

Cell counts. After fixation of samples in 0.5% neutralized glutaraldehyde in autoclaved and filtered seawater (0.2-μm-pore-size Gelman Acrodisc), they were stained with 0.01% acridine orange and counted by epifluorescence microscopy (9).

Electron microscopy. For transmission electron microscopy, cells were fixed (glutaraldehyde and osmium tetroxide) and stained (uranyl acetate and lead citrate) by the method of Rippkä et al. (18). Thin sections were examined with a Zeiss EM-109 transmission electron microscope. For scanning electron microscopy, the cells were fixed and treated as described by Jannasch and Wirsen (10) and were examined with a JEOL JSM-840 scanning electron microscope.

DNA extraction and base composition. DNA was extracted, the thermal denaturation (T_m) was determined, and the

* Corresponding author.

† Contribution no. 6061 of the Woods Hole Oceanographic Institution.

moles percent G+C was calculated by the methods of Herdman et al. (8).

Muramic acid analysis. Cells from the exponential growth phase (ca. 30 mg [wet weight]) were washed once with 1 N HCl and then hydrolyzed for 30 min at 100°C in 5 ml of 4 N HCl. The extract was dried three times, suspended in distilled water, and filtered (0.2-μm-pore-size Gelman Acrodisc). Fluorescent derivitization with *o*-phthaldialdehyde was carried out by the method of Lindroth and Mopper (14). Samples (15 μl) were analyzed by high-pressure liquid chromatography as described by Lee and Cronin (13), using an Ultrasphere-ODS 5-μm column (25 cm by 4.6 mm; Altex Scientific Co.) and an FS970 fluorescence detector. Muramic acid eluted between glutamic acid and serine and was identified by retention time and coinjection with the pure compound.

Sulfide determination. Sulfide was determined by the method of Cline (6), with slight modifications. A sample (0.08 ml) of the *N*,*N*-dimethyl-*p*-phenylenediamine sulfate reagent was introduced into a nitrogen-flushed 1-ml syringe. The volume was then brought to 1 ml with the sample, which was withdrawn through the rubber septum of the Hungate tube. When the sulfide concentration exceeded 1 mM, the sample was first diluted with oxygen-free distilled water. Absorbance of methylene blue was determined at 670 nm after a 30-min development period and appropriate dilution. Sulfide concentrations were calculated from standard curves.

Glucose consumption. Glucose was determined with a Worthington Statzyme Glucose reagent (Cooper Biomedical, Inc., West Chester, Pa.).

RESULTS

Morphology and ultrastructure. Exponentially growing cells of strain NS-E at 77°C exhibited diverse morphological characteristics in different growth media. When glucose was used as a single carbon source supplemented with a small amount of yeast extract, the cells appeared as regular, single rods, approximately 0.5 by 2 μm (Fig. 1a and b). Although growth rates on marine broth were the same, the rods were somewhat enlarged and appeared half empty, with the cytoplasm concentrated at the center of the cell (Fig. 1c and d). Growth at high glucose concentration (25 g liter^{-1}) resulted in initial elongation of the cells, some of which exceeded 100 μm in length. After 24 h, however, the long cells divided into smaller rods, presumably in response to the decrease in glucose concentration.

Examination of thin cross sections of the regular rod-shaped cells revealed an unusually thick periplasmic cell wall layer (Fig. 1b). While lacking the characteristic structure of gram-negative cell walls, this layer is covered with a more electron-dense outer layer. This wall unit retains its integrity when the cytoplasm has shrunken away (Fig. 1d).

DNA base composition. The molar ratio of guanine plus cytosine (G+C) in the DNA of strain NS-E was calculated from T_m measurements to be 41.3 mol%.

Eubacterial properties. Growth of strain NS-E was completely inhibited by 100 μg of chloramphenicol, vancomycin, or streptomycin ml^{-1} but not by rifampin at the same concentration. Hydrochloric acid hydrolysates of the cells contained muramic acid at approximately 1 μg (dry weight) mg^{-1}. This value is lower than reported for other eubacteria (12; D. White, personal communication) but is comparable to values we obtained for some eubacterial control species by using the same procedure.

The presence of muramic acid as well as the specific drug sensitivity are indications that strain NS-E is a eubacterium (31). The insensitivity to rifampin may be an inherent property of this strain or may be due to permeability problems. It cannot be attributed to thermal inactivation of the antibiotic, since growth of other thermophilic isolates was completely blocked in the presence of this drug at the same temperature.

Temperature and pH optima. Strain NS-E grew between 50 and 95°C, with optimum generation times of 40 to 45 min at 77°C (Fig. 2A). The pH optimum for growth was 7.5 (Fig. 2B). Growth rates at pH 6 and 9 were 50% of the optimum value.

Nutritional requirements. Strain NS-E appears to be an obligate heterotroph. No growth could be detected after 7 days in mineral media with various combinations of electron donors (HS^-, S^0, H_2, $S_2O_3^{2-}$) and acceptors (S^0, SO_4^{2-}, NO_3^-, NO_2^-) in the presence of 20% (vol/vol) CO_2 in an N_2 or H_2 gas phase.

Strain NS-E grew well on half-strength marine broth (2.5 g of peptone liter^{-1} and 0.5 g of yeast extract liter^{-1}) and on yeast extract alone (2.5 g liter^{-1}). Of the single carbon sources tested, only sugars (glucose, sucrose, lactose, and starch) supported growth. Generation times with yeast extract, glucose, glucose plus yeast extract, or in marine broth were similar, around 40 to 45 min. Final cell yields with additional glucose were higher than those attained during growth on yeast extract alone or on marine broth.

No growth could be detected after 48 h with the following carbon sources (2 g liter^{-1} each): acetate, lactate, formate, pyruvate, propionate, mannitol, ethanol, methanol, glycerol, glutamate, or glycine. Growth on peptone or tryptone was weak, and no growth was measured with Casamino Acids (Difco).

Sulfur reduction. In the presence of elemental sulfur, growth of strain NS-E was accompanied by exponential sulfide production which exactly paralleled growth (Fig. 3). In the yeast extract-glucose medium, the addition of sulfur did not affect growth rates but reproducibly enhanced final cell yield up to fourfold. With glucose alone (2 g liter^{-1}), final cell densities were similarly increased by the presence of sulfur (from 2×10^8 to around 5×10^8 cells ml^{-1}). In all cases, sulfur reduction to sulfide was a biological process, i.e., it did not occur when growth was inhibited by chloramphenicol or when a nonutilizable carbon source such as acetate was used. Sulfide production did not occur in the absence of cells. As observed earlier (3), 77°C is just below the minimal temperature required for the abiological conversion of sulfur to sulfide.

Sulfide was not produced when SO_4^{2-} or $S_2O_3^{2-}$ was substituted for elemental sulfur, and neither did the presence of these compounds affect the growth parameters of strain NS-E.

Sulfide toxicity. During growth in the presence of elemental sulfur, sulfide concentrations as high as 10 mM were regularly attained (Fig. 3). Lowering of this concentration by flushing with N_2, which removed most of the sulfide from the gas phase of the culture, resulted in the immediate resumption of growth. It stopped again when the sulfide concentration reached about 6 mM. This treatment allowed for a doubling of the final cell density (Fig. 3).

To determine the inhibitory sulfide level for strain NS-E, cultures were incubated in the presence of various sulfide concentrations. In Fig. 4 growth after 24 h is presented as the percentage of a sulfide-free control. In the presence of 1 mM sulfide growth was inhibited by about 65%, and at 10 mM sulfide it was inhibited by over 95%. No growth occurred when 20 mM sulfide was initially present.

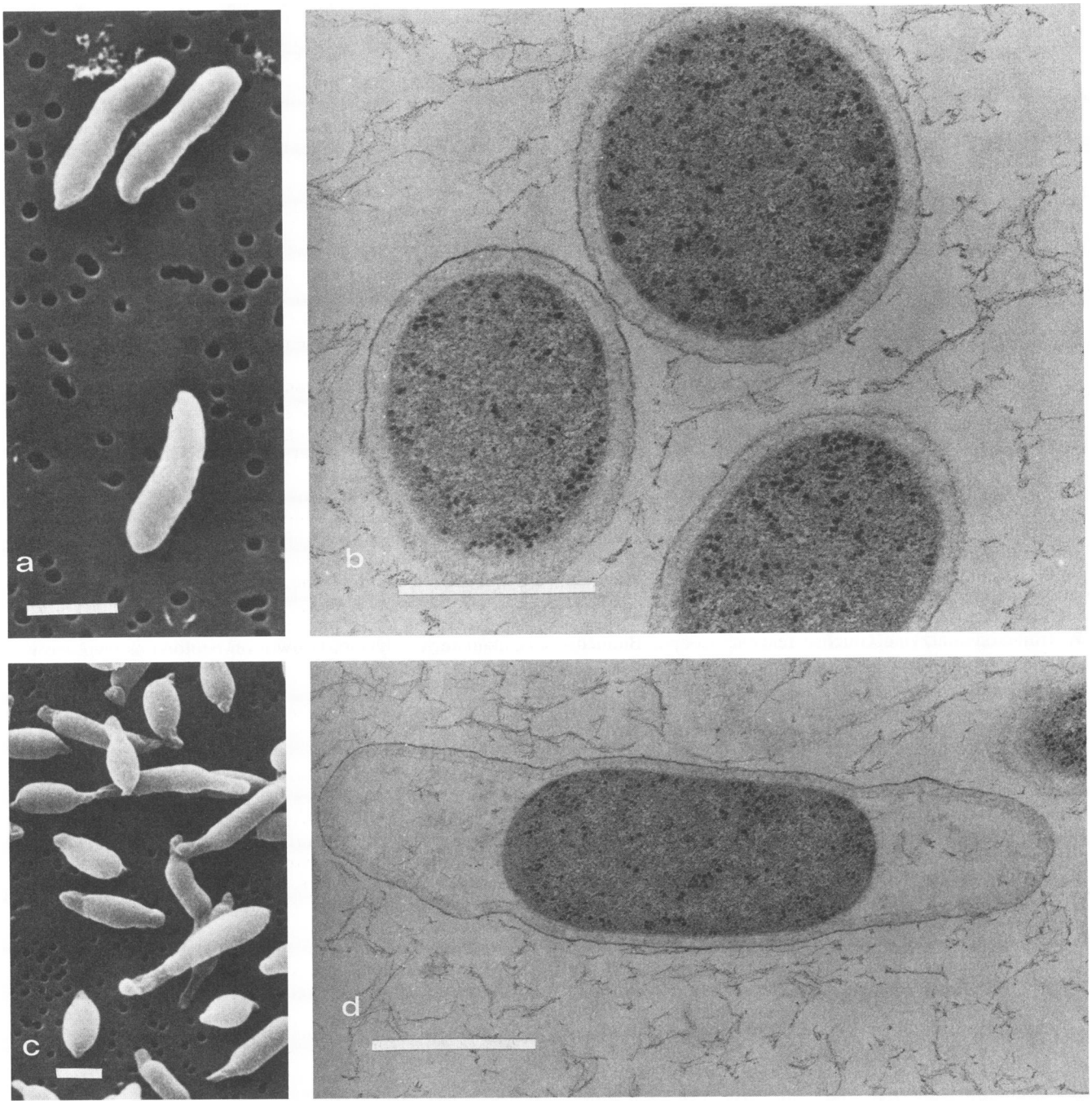

FIG. 1. Scanning (SEM) and transmission (TEM) electron micrographs of strain NS-E: SEM (a; scale bar, 1 μm) and TEM cross-section (b; scale bar, 0.5 μm) of cells grown in glucose medium (1 g liter^{-1}) with yeast extract (0.1 g liter^{-1}); SEM (c; scale bar, 1 μm) and TEM longitudinal section (d; scale bar, 0.5 μm) of cells grown in half-strength 2216 marine broth medium.

Glucose utilization. To clarify the effect of sulfur respiration on the metabolism of strain NS-E, the consumption of glucose was followed in cultures grown with or without the addition of elemental sulfur (Fig. 5). Both cultures grew at similar rates through the 6-h exponential growth phase. At that time chemical analyses showed that each culture had equivalent glucose consumption per cell (2.8×10^{-8} μmol of glucose cell^{-1}) or per unit protein (2.0×10^{-2} μmol of glucose μg of protein^{-1}). However, after the culture without added sulfur approached the stationary phase (8 to 24 h), a distinct difference became apparent. The sulfur-containing culture continued to grow and utilized approximately 50% more of the available glucose. Even at that stage the amount of glucose consumed per cell did not vary significantly. Comparison of the amounts of sulfide produced with the amounts of glucose consumed revealed a ratio of approximately 1.0. Taking into consideration the amounts of sulfide present in the gas phase of the culture tubes (as compared with the sulfide concentration in the liquid phase actually measured), this ratio can be increased up to twofold, i.e., about 2 mol of sulfide are generated per mol of glucose consumed.

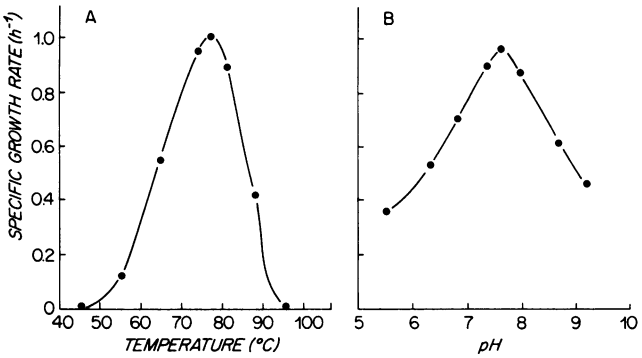

FIG. 2. Growth of strain NS-E as a function of temperature and pH, determined in half-strength 2216 marine broth medium. (A) Temperature optimum (20 mM PIPES buffer at pH 7.5); (B) pH optimum (20 mM MES [morpholineethanesulfonic acid] or bis-tris propane buffers for pHs below or above 6.5, respectively).

Oxygen and temperature tolerance. Cells of strain NS-E are insensitive to oxygen below those temperatures at which growth occurs. Cultures have been stored for several months at 4°C under air with no significant loss of viability. Above 50°C, exposure to air leads to cell mortality, which is very fast at 77°C. Above 95°C, in the presence as well as in the absence of air, the cells lyse after short incubation periods.

DISCUSSION

Isolate NS-E is an obligately anaerobic, extremely thermophilic heterotrophic eubacterium. It may also be defined as caldoactive according to the definition of Williams (30) since its growth temperature minimum is above 40°C and its growth optimum is above 65°C. The data presented indicate that it is phenotypically distinct from the following extreme thermophilic, obligately anaerobic, nonsporeforming, rod-shaped eubacteria: *Thermoanaerobacter ethanolicus* (29), *Thermoanaerobium brockii* (34), *Thermobacteroides acetoethylicus* (4), and *Thermodesulfobacterium commune* (33). The criteria are: (i) the optimum growth temperature of isolate NS-E is considerably higher (77°C as compared with 65 to 70°C); (ii) it is of marine origin; all other strains are isolated from terrestrial hot springs; (iii) its DNA base composition (41.3 mol%) is higher than that reported for *Thermoanaerobium brockii* (30 to 31.4 mol%), *Thermodesulfobacterium commune* (34.4 mol%), or *Thermobacteroides acetoethylicus* (31.1 mol%); it is near the value reported for *Thermoanaerobacter ethanolicus* (37 to 39 mol% from buoyant density), although a different value (31.9 mol%) has also been obtained for that organism from thermal denaturation studies (29); (iv) its morphological variability is unusual; only *Thermoanaerobacter ethanolicus* resembles NS-E by cell elongation and spheroplastlike forms observed in stationary cultures; (v) it can grow on yeast extract or glucose alone, unlike *Thermoanaerobacter ethanolicus* and *Thermoanaerobium brockii*; (vi) it is unflagellated unlike *Thermoanaerobacter ethanolicus* and *Thermobacteroides acetoethylicus*; (vii) its generation time (40 to 45 min) is much shorter than that of *Thermodesulfobacterium commune* (about 4 h); (viii) unlike the latter, it does not utilize pyruvate, lactate, or H_2 or reduce sulfate or thiosulfate; and (ix) so far, it is the only eubacterial extreme thermophile reported to reduce elemental sulfur.

In a recent paper, Stetter (22) mentions the isolation of a fermentative organism (strain MSB8) from a source similar to that from which strain NS-E was obtained. Strain MSB8 also shows a very similar morphology and range of growth temperature, but no capacity of sulfur reduction is indicated. Although the brief description is part of a discussion on archaebacteria, the isolate MSB8 is not specifically defined as such.

The ability of strain NS-E to reduce elemental sulfur deserves special notice. This mode of respiration has been described for a few eubacterial mesophiles (17, 32) and many archaebacterial thermophiles (2, 7, 23, 24, 35–38). We have recently suggested that thermophilic sulfide production from sulfur is a temperature-related phenomenon (3). As mentioned above, strain NS-E is the only extremely thermophilic eubacterial isolate to date shown to carry out this reaction. Thermophilic sulfur reduction may be characteristic of eubacterial as well as archaebacterial thermophiles.

When growing in the absence of sulfur, strain NS-E derives its energy probably at the substrate level. As in other fermentative bacteria (27), this can still allow for relatively fast growth. The addition of elemental sulfur is obviously advantageous since, even though growth rates do not increase, higher cell desities are reached. The metabolic mechanism by which this effect is achieved is not clear. A simple shift from fermentative to respiratory metabolism is unlikely because: (i) specific growth and glucose utilization rates are equal in the presence or absence of sulfur; (ii) the amount of glucose consumed per cell or per unit protein does

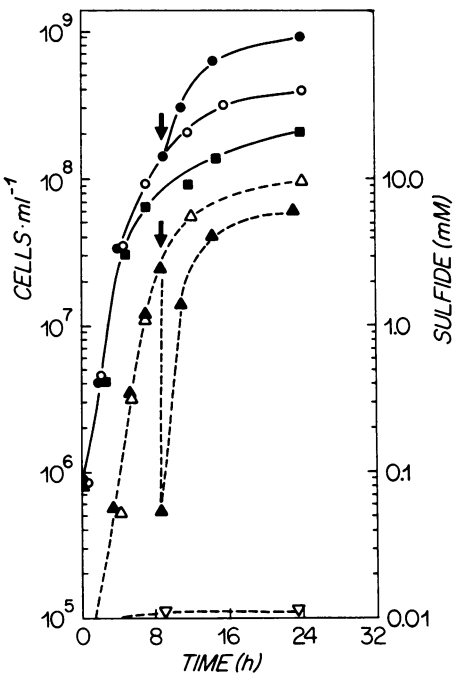

FIG. 3. Growth and sulfide production of strain NS-E in artificial seawater medium with glucose (2 g liter^{-1}), yeast extract (0.5 g liter^{-1}), and PIPES buffer (20 mM) at pH 7.5: Symbols: ■, growth in the absence of sulfer; ○, growth in the presence of sulfur; ●, growth in the presence of sulfur with removal of the sulfide from the gas phase after 9 h (arrow); △, sulfide production in the presence of sulfur; ▲, sulfide production in the presence of sulfur with removal of sulfide from the gas phase after 9 h (arrow); ▽, sulfide production in uninoculated sulfur-containing medium or in inoculated medium with chloramphenicol (100 μg ml^{-1}), or with acetate (2 g liter^{-1}) as the sole carbon source.

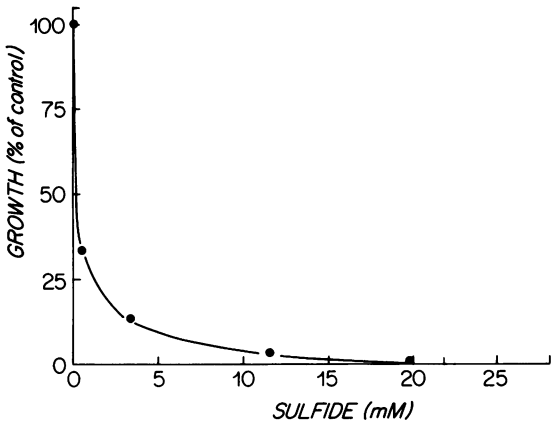

FIG. 4. Sulfide inhibition of growth of strain NS-E. Cell densities of strain NS-E after 24 h of incubation in the presence of various sulfide concentrations presented as percentage of the sulfide-free control (stock sulfide solutions were titrated to pH 7.5 before addition to culture tubes).

not vary; (iii) the ratio of sulfide produced per glucose used is, at most, 2:1. Theoretically complete respiratory degradation of glucose would result in a ratio of 12:1. The nature of the enhancing effect of elemental sulfur is under examination.

It is remarkable that growth of strain NS-E, a very effective sulfide producer, is inhibited by relatively low sulfide concentrations. However, sulfide is unlikely to reach those high concentrations in the natural habitat of this organism. Furthermore, the occurrence of this organism in hydrothermal systems indicates that not all of the hydrogen sulfide found in vent water is of geothermal origin but may also be produced biologically. This assumption will have to be checked by stable sulfur isotope determinations. Finally, the capability of strain NS-E to remain viable for long periods of time under oxygenated conditions at low temperatures appears to be an important trait for its survival in these confined and insular habitats and for its transport to newly emerging vent sites.

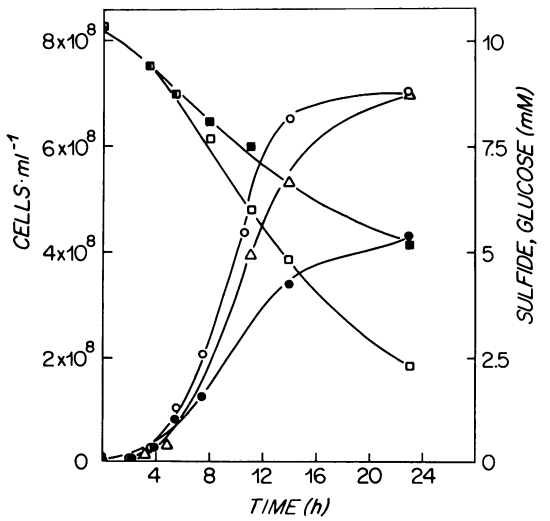

FIG. 5. Glucose utilization and sulfide production by strain NS-E. Growth in artificial seawater medium with glucose (2 g liter^{-1}), yeast extract (0.5 g liter^{-1}), and PIPES buffer (20 mM), pH 7.5. Symbols: ○, growth in the presence of elemental sulfur; ●, growth in the absence of elemental sulfur; □, glucose consumption in the presence of sulfur; ■, glucose consumption in the absence of sulfur; △, sulfide production in the presence of sulfur.

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We thank C. Lee and C. Cronin for conducting the analyses for muramic acid.

ADDENDUM IN PROOF

After this paper had gone to press, we received a manuscript by R. Huber, T. A. Langworthy, H. Konig, M. Thomm, C. R. Woese, U. B. Sletyr, and K. O. Stetter, entitled "*Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C" (Arch. Microbiol., in press). This article describes strain MSB8; the name specifically addresses its peculiar morphology. It is most likely that strain NS-E is a member of this new genus, although it may represent a different species.

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